

Two glucose/xylose transporter genes from the yeast *Candida intermedia*: first molecular characterization of a yeast xylose–H⁺ symporter

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Candida intermedia PYCC 4715 was previously shown to grow well on xylose and to transport this sugar by two different transport systems: high-capacity and low-affinity facilitated diffusion and a high-affinity xylose–proton symporter, both of which accept glucose as a substrate. Here we report the isolation of genes encoding both transporters, designated *GXF1* (glucose/xylose facilitator 1) and *GXS1* (glucose/xylose symporter 1) respectively. Although *GXF1* was isolated by functional complementation of an *HXT*-null (where *Hxt* refers to hexose transporters) *Saccharomyces cerevisiae* strain, isolation of the *GXS1* cDNA required partial purification and micro-sequencing of the transporter, identified by its relative abundance in cells grown on low xylose concentrations. Both genes were expressed in *S. cerevisiae* and the kinetic parameters of glucose and xylose transport were determined. *Gxs1* is the first yeast xylose/glucose–H⁺ symporter to be characterized at the molecular level. Comparison of its amino acid sequence with available sequence data revealed the existence of a family of putative monosaccharide–H⁺ symporters encompassing proteins from several yeasts and filamentous fungi.

Key words: *Candida intermedia*, membrane protein, sugar transport, xylose/glucose–H⁺ symporter.

INTRODUCTION

Ethanol that is to be used as fuel is currently produced through fermentation of glucose-rich hydrolysates derived from dedicated agricultural crops (e.g. corn and wheat). However, the use of hemicellulosic materials as feedstock is deemed preferable both from an environmental and from an economic point of view because hemicellulose is a major component of low-value agricultural and wood-pulping wastes [1,2]. Ideally *Saccharomyces cerevisiae*, as the most efficient glucose fermenter and capable of withstanding both high ethanol concentrations and the presence of toxic compounds in hemicellulose hydrolysates, would also be used to convert the pentose fraction in these hydrolysates into ethanol. However, *S. cerevisiae* does not utilize xylose, which is the predominant pentose in hemicellulose, as a carbon and energy source. To circumvent this obstacle, the genes encoding two enzymes that are required for xylose catabolism [XDH (xylose dehydrogenase) and XR (xylitol reductase)] were cloned from the xylose fermenter *Pichia stipitis* and successfully expressed in *S. cerevisiae* [3].

S. cerevisiae also lacks an efficient transport system for xylose although the sugar can enter the cell via the non-specific *HXT* hexose transporters [4], albeit with very low affinity (K_m approx. 100 mM). A comprehensive quest for a high-affinity xylose–H⁺ symporter is justified by the prospect of improving the performance of currently available recombinant *S. cerevisiae* xylose-fermenting strains [5,6]. Nevertheless, attempts to isolate such genes have, so far, been unsuccessful [7].

The yeast *Candida intermedia* PYCC 4715 grows well in xylose medium and shows high xylose-transport capacity. *C. intermedia* has both high- and low-affinity transport systems for xylose and is a potentially interesting gene donor for the heterologous expression of an efficient xylose transporter in *S. cerevisiae*. Biochemical characterization of xylose and glucose transport in

C. intermedia PYCC 4715 showed the facilitated diffusion mechanism to be constitutive (K_m approx. 50 mM for xylose), whereas the glucose/xylose–H⁺ symporter (K_m approx. 0.2 mM for xylose) increased its capacity by one order of magnitude in low-xylose medium and was absent from batch glucose-grown cells [8].

In the present paper, the identification of genes encoding xylose/glucose transporters from *C. intermedia* is described. Several approaches were followed to identify and characterize these genes. In addition to functional complementation, strategies exploring the regulation of the glucose/xylose–H⁺ symporter by the carbon source were used. The isolated genes were expressed in *S. cerevisiae* and their biochemical properties were determined. Molecular characterization of the glucose/xylose–H⁺ symporter from *C. intermedia* unveiled a group of putative, previously uncharacterized, fungal monosaccharide–H⁺ symporters, comprising proteins from several different yeasts and filamentous fungi.

EXPERIMENTAL

Yeast strains and growth conditions

The strains used or generated in this work were: *C. intermedia* PYCC 4715 (Portuguese Yeast Culture Collection, Caparica, Portugal), *S. cerevisiae* TMB 3201 (*MATa Δhxt1-17 Δgal2 Δstl1 Δagt1 Δmph2 Δmph3 leu2-3,112 ura3-52 trp1-289 his3-Δ1::YlpXR/XDH/XK MAL2-8° SUC2*; [9]), MJY1 (TMB 3201 + pGXF1), MJY2 (TMB 3201 + pHXT7-GXS1), MJY3 (TMB 3201 + pPGK-GXS1), MJY4 (TMB 3201 + pHXT7-GXS1-s) and MJY5 (TMB 3201 + pPGK-GXS1 + pGXF1) (GXF, glucose/xylose facilitator; GXS, glucose/xylose symporter; PGK, phosphoglycerate kinase). Yeast strains were routinely grown in YNB (Difco yeast nitrogen base without amino acids) medium containing the indicated carbon source and the required supplements, at 25 °C and 150 rev./min, unless otherwise stated. Strain

Abbreviations used: GXF, glucose/xylose facilitator; GXS, glucose/xylose symporter; ORF, open reading frame; PGK, phosphoglycerate kinase; RACE, rapid amplification of cDNA ends; RLM-RACE, RNA ligase-mediated RACE; RT, reverse transcriptase; XDH, xylose dehydrogenase; XR, xylitol reductase.

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The DNA sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AJ937350 and AJ875406.

TMB 3201 and plasmid pMA91 were provided by Professor Bärbel Hahn-Hägerdal, University of Lund, Sweden.

Construction and screening of a *C. intermedia* genomic library

A genomic library from *C. intermedia* PYCC 4715 was constructed as follows. Genomic DNA was partially digested with Sau3A and the resulting DNA fragments were separated in 10–40 % (w/v) discontinuous sucrose gradients. Fractions containing fragments in the ranges 4.5–11 kb, 6–12 kb and 8–13 kb were pooled and used in separate ligations. Yeast shuttle vector YEplac195 [10], digested with BamHI and dephosphorylated, was ligated to the purified genomic DNA fragments. Epicurian Coli XL10-GOLD ultracompetent cells (Stratagene) were transformed with the ligations and plated on to LB (Luria–Bertani) medium supplemented with 75 mg/l ampicillin, 40 µg/ml X-Gal (5-bromo-4-chloroindol-3-yl β-D-galactopyranoside) and 40 µg/ml IPTG (isopropyl β-D-thiogalactoside), yielding approx. 11 200 white colonies. Restriction analysis of plasmid DNA isolated from 50 white colonies revealed an average insert length of 3.5 kb. Library transformants were washed from the plates with LB medium, glycerol stocks were prepared, and the remaining cell suspension was allowed to grow for one generation in the presence of ampicillin. The cells were then harvested and plasmid DNA was isolated.

S. cerevisiae strain TMB 3201 was transformed with library plasmid DNA using the lithium acetate method [11]. The transformation mixture was first plated on to solid YNB medium with 2 % maltose as the sole carbon source. The transformants obtained were then transferred by replica plating on to the same YNB medium containing either xylose or glucose at the concentrations indicated.

Sugar-transport assays

Initial D-[¹⁴C]glucose and D-[¹⁴C]xylose (Amersham Biosciences) uptake rates were measured as described previously [12]. Cultures were harvested at a D_{640} of approx. 0.7. Each experiment was performed at least twice. Symport activity was assessed by the proton influx elicited by the addition of glucose or xylose to an aqueous cell suspension. pH data were collected from a pHM240 pH/ion-meter (MeterLab) and recorded by computer, using custom-made software.

Nucleotide sequences and accession numbers

To facilitate sequencing of the relevant portion of the *C. intermedia* genomic DNA insert in plasmid pGXF1, 1.3 kb and 1.4 kb EcoRI fragments, as well as a 3.2 kb KpnI fragment from plasmid pGXF1, were cloned into vector pUC18, originating plasmids pGXFE1, pGXFE2 and pGXFK1 respectively. Plasmid pGXFE2 was digested with HindIII, releasing a fragment of 200 bp, and was re-ligated, originating plasmid pGXFE2H. The inserts on these four plasmids were sequenced using an ALFexpress™ II DNA Analyser (Amersham Biosciences), and 5'-Cy5-labelled vector-specific primers (Thermo Sequenase Primer Cycle Sequencing kit). The DNA sequence of the *GXF1* ORF (open reading frame) is available from the EMBL (European Molecular Biology Laboratory) database under accession number AJ937350.

The two pMOSBlue clones containing the 5' and 3' RACE (rapid amplification of cDNA ends) fragments from the *GXS1* gene (see below) were used to obtain the sequence of the gene. The DNA sequence of the *GXS1* ORF is available from the EMBL database under accession number AJ875406.

Isolation of yeast plasma membranes

C. intermedia PYCC 4715 cells were harvested at the exponential growth phase ($D_{640} = 0.8$ – 2.0) in Verduyn medium [13] containing either 0.5 % (w/v) xylose, 2 % (w/v) glucose or 4 % (w/v) xylose, and washed twice with ice-cold distilled water and once with buffer A (0.1 M glycine, 0.3 mM KCl, pH 7.0). Cells (10–15 g) were subsequently re-suspended in 15 ml of buffer A containing 0.1 mM PMSF. Plasma membranes were isolated further as described by Van Leeuwen et al. [14]. Protein concentrations were determined using the Lowry assay [15], with BSA as a standard.

Tricine SDS/PAGE of plasma-membrane proteins and microsequencing

Aliquots of isolated plasma and mitochondrial membranes containing 20 µg of total protein were separated by Tricine SDS/PAGE [16], in a 10 % polyacrylamide gel (with 0.3 % bisacrylamide). In the preparative gel (7.5 % polyacrylamide, with 0.23 % bisacrylamide), 250 µg of total protein from plasma membranes of *C. intermedia* cells cultivated in 0.5 % (w/v) xylose was separated (Protein II xi Cell apparatus, Bio-Rad), following the addition of 11.4 mg/l thioglycolate to the cathode buffer (to scavenge reactive compounds left in the gel, which may cause N-terminal blocking of the proteins), according to the manufacturer's instructions for the Sequi-Blot PVDF membrane for protein sequencing (Bio-Rad). The gel was subsequently blotted on to a PVDF membrane, according to the manufacturer's instructions, using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) apparatus and buffers for electroblotting previously described by Schägger [16] for Tricine-SDS gels. The protein band of interest was then excised from the blot and its N-terminus sequenced by the Protein Core Facility, Howard Hughes Medical Institute at Columbia University, NY, U.S.A.

Cloning of the complete *GXS1* cDNA

The cDNA encoding the putative glucose/xylose symporter was obtained by RACE using the FirstChoice® RLM (RNA ligase-mediated)-RACE kit (Ambion). Total RNA was extracted from *C. intermedia* PYCC 4715 cells cultivated in 0.5 % (w/v) xylose and purified using the RNACleanup protocol in the RNeasy kit (Qiagen). This RNA sample was used as a template for the 3' RLM-RACE protocol, in combination with gene-specific degenerate primers CiGXSL1 (5'-GARGAYAAAYMGIATGGTIA-ARMG-3') and CiGXSL2 (5'-AARMGITTGYGTIAAYGTNGG-3'). The design of these primers was based on the amino acid sequence (see Figure 4) obtained for the putative glucose/xylose symporter. Gene-specific primer CiGXSR3 (5'-CGTTAAGGA-ATGGAGCACAAAG-3') was used in the 5' RACE reaction. Amplified fragments were cloned into the pMOSBlue vector (Amersham Biosciences) and sequenced using an ALFexpress™ II DNA Analyser (Amersham Biosciences), and 5'-Cy5-labelled vector-specific primers (Thermo Sequenase Primer Cycle Sequencing kit).

Northern blot

Before RNA isolation, the cells of *C. intermedia* PYCC 4715 were frozen in liquid nitrogen. Total RNA isolations and gel electrophoresis were performed as described previously [17]. The gels were blotted on to Hybond-N Nylon membranes (Amersham Biosciences). A 163 bp fragment of the *GXF1* gene, amplified from plasmid pGXF1 with primers CiGXFL1 (5'-CTTTGCTT-CCACCTTCGTTG-3') and CiGXFR1 (5'-AGTGTGGAGGT-CTCGTTGG-3'), was used as a *GXF1*-gene-specific probe. A 300 bp fragment amplified from a plasmid containing the first

400 bp of the *GXS1* gene with primers CiGXSL1 and CiGXSR3 was used as a *GXS1*-gene-specific probe. A 172 bp fragment of the actin gene, amplified from genomic DNA of *C. intermedia* PYCC 4715 with primers ActCiL1 (5'-AACAGAGAGAAGATGACC-CAGA-3') and ActCiR1 (5'-GCAAAGAGAAACCAGCGTAA-3'), was used as an actin-gene-specific probe. Probes were labelled with [α - 32 P]ATP (Amersham Biosciences) using the Prime-a-Gene Labelling system (Promega). Hybridization and washing conditions were as described previously [17].

Plasmid construction

A DNA fragment encompassing nucleotides -392 to -1 of the *HXT7* promoter was amplified by PCR using plasmid YEpkHXT7 [18] as a template and primers HXT7prom1 (5'-AACCTGCA-GCTCGTAGGAACAATTCGG-3') and HXT7prom2 (5'-GG-ACGGGACATATGCTGATTAATAAATAAAAACTT-3'). At the 5' end these two primers contain restriction sites for PstI and NdeI respectively. The complete *GXS1* ORF was amplified using primers GXS1P1 (5'-ATAGCAGATCTCATATGGGTTTGGAG-GACAATAGAATG-3') and GXS1P2 (5'-ATAGCAGATCTTCT-AGATTAAACAGAAGCTTCTTCAGAC-3') and genomic DNA of *C. intermedia* as a template. Both primers contain, at the 5' end, a BglII restriction site and, in addition, an NdeI (GXS1P1) or XbaI (GXS1P2) restriction site. Plasmid YEplac195 [10] was digested with PstI and XbaI and ligated to the *HXT7* promoter fragment, previously digested with PstI and NdeI, and to the *GXS1* ORF fragment, previously digested with NdeI and XbaI, resulting in plasmid pHGXS1. A 0.3 kb fragment of the terminator region of the *PGK* gene was amplified using primers PGK1term1 (5'-AC-CGTGTCTAGATAAATTGAATTGAATTGAAATCGA TAG-3') and PGK1term2 (5'-TAATTAGAGCTCTCGAAAGCTTTAA-CGAACGCAGAA-3') and plasmid pMA91 [19] as a template. The primers contain, at the 5' end, restriction sites for XbaI and SacI respectively. The *PGK* terminator fragment was subsequently cloned between the XbaI and SacI restriction sites in plasmid pHGXS1, originating plasmid pHXT7-GXS1.

Plasmid pMA91 [19] was digested with BglII and ligated to the *GXS1* ORF fragment, amplified as described above and digested with BglII, resulting in plasmid pPGK-GXS1.

Plasmid pHXT7-GXS1 was digested with PstI and SacI to release the HXT7(promoter)-GXS1(ORF)-PGK(terminator) insert that was then cloned into centromeric plasmid YCplac111 [10], between the PstI and SacI sites, resulting in plasmid pHXT7-GXS1-s.

Miscellaneous

Molecular biology techniques were performed using standard protocols [20]. Restriction enzymes and ligase were purchased from Roche. *Escherichia coli* strains DH5 α (New England Biolabs), Sure (Stratagene), Epicurian Coli XL10-GOLD ultra-competent cells (Stratagene) and MOSBlue (Amersham Biosciences) were used as hosts for DNA manipulations. Primers were obtained from MWG Biotech (Ebersberg, Germany).

RESULTS

Isolation of a *C. intermedia* glucose/xylose transporter gene by functional complementation

A *C. intermedia* PYCC 4715 genomic library, constructed in *S. cerevisiae* shuttle vector YEplac195 (*URA3*), was used to transform an *S. cerevisiae* strain devoid of glucose transporters but carrying the heterologous genes required for xylose catabolism (strain TMB 3201; [9]). This strain has previously been shown to

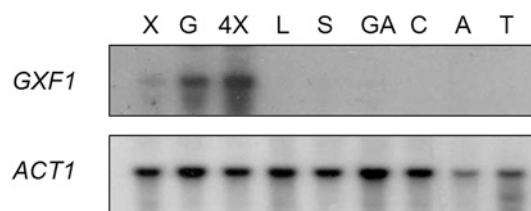


Figure 1 Northern blot analysis of *GXF1* expression in *C. intermedia* PYCC 4715 cells cultivated in different carbon sources

The carbon sources (w/v) were: 0.5 % xylose (X), 2 % glucose (G), 4 % xylose (4X), 0.5 % lactose (L), 0.5 % sorbose (S), 0.5 % galactose (GA), 0.5 % cellobiose (C), 0.5 % (w/v) arabinose (A), or 0.5 % trehalose (T). Northern blots were probed with a *GXF1*-gene-specific probe and, subsequently, with an actin-gene-specific probe, as a loading control.

be unable to transport either glucose or xylose [9]. Ura⁺ transformants were first recovered in YNB maltose medium and subsequently transferred by replica plating on to medium containing different concentrations of either xylose or glucose as the sole carbon and energy source. Approx. 40 transformants that were able to grow well on glucose, although they grew weakly on xylose, were selected. None of the transformants exhibited proton symport activity, as determined by the absence of proton influx movements elicited by the addition of either glucose or xylose to an aqueous cell suspension. For a subset of the transformants, it could be shown readily that growth on glucose and on xylose was dependent on library plasmids. A plasmid carrying an 11 kb *C. intermedia* genomic-DNA insert (pGXF1) was recovered from one of the transformants and used to re-transform the host strain. The phenotype of the resulting transformants was similar to that observed for the original library clone (results not shown). One of these transformants (strain MJY1) was selected for further characterization. Subcloning and subsequent sequencing revealed the presence of an ORF encoding a putative membrane protein of 547 amino acids. Similarity and domain searches revealed signature sequences found in all sugar transporters and a strong similarity (66–65 % identity) with the *SUT1*–*SUT3*-encoded glucose facilitators from *P. stipitis* [21]. Transcriptional regulation of the new gene was studied by Northern blot, using total RNA from *C. intermedia* cells grown on different carbon sources. The gene was expressed in cells cultivated either on glucose or on xylose, but not on the other carbon sources tested (Figure 1). The combined evidence suggested that plasmid pGXF1 encodes a *C. intermedia* glucose/xylose facilitator.

This was supported by D-[14 C]xylose- and D-[14 C]glucose-uptake experiments (Figure 2). The novel transporter displayed a much higher affinity for glucose ($K_m = 2.0 \pm 0.6$ mM) than for xylose ($K_m = 48.7 \pm 6.5$ mM). It is noteworthy that the K_m values are very similar to those determined for the glucose/xylose-facilitated diffusion transport system in *C. intermedia* PYCC 4715 cells [8]. The novel gene was named *GXF1*.

Identification of the glucose/xylose-H⁺ symporter by SDS/PAGE

Several rounds of screening of the *C. intermedia* genomic library failed to yield transformants exhibiting sugar-H⁺-symport activity. Therefore different strategies were devised to clone the gene encoding the symporter, taking advantage of the fact that symport activity, and presumably expression of the corresponding gene, increases by approx. 8-fold in *C. intermedia* cells cultivated in low-xylose media, being absent in cells grown in high glucose concentrations [8]. First, cDNA fragments encoding sugar transporters expressed in a low xylose concentration medium were recovered using RT (reverse transcriptase)-PCR with degenerate

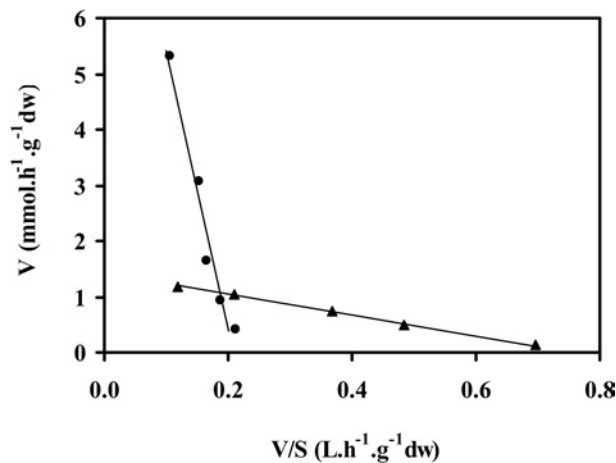


Figure 2 Glucose and xylose uptake in strain MJY1 (*S. cerevisiae* TMB 3201 transformed with pGXF1)

Eadie-Hofstee plots of initial uptake rates of (●) D-[¹⁴C]xylose and (▲) D-[¹⁴C]glucose by strain MJY1 cultivated on glucose. dw, dry weight.

primers based on amino acid stretches conserved among all fungal sugar transporters. This approach yielded two cDNA fragments corresponding to putative sugar transporters, as shown by partial sequencing (results not shown). However, Northern blot analysis revealed an expression pattern of the concomitant genes that did not agree with the available data on the regulation of xylose-H⁺-symport activity (results not shown).

The second approach was directed to plasma-membrane proteins. It consisted of the identification of the envisaged transporter by a comparison of the relative abundance of membrane proteins in plasma membranes isolated from *C. intermedia* cells cultivated under inducing and repressing conditions. To this end, plasma-membrane preparations and the corresponding mitochondrial-membrane fractions, isolated from cells cultivated in 0.5% (w/v) xylose, 2% (w/v) glucose or 4% (w/v) xylose, were run on SDS/PAGE (10% polyacrylamide gel, with 0.3% bisacrylamide) optimized for the separation of membrane proteins. Plasma membranes exhibited band patterns that were clearly different from those of the corresponding mitochondrial membranes (Figure 3), indicating that separation of the two types of membranes was effective. A comparative analysis of protein patterns from the three plasma-membrane preparations (Figure 3) showed an obvious band corresponding to a protein with a molecular mass of approx. 40 kDa, which appeared to be present only in plasma membranes isolated from cells grown in 0.5% (w/v) xylose. Since the molecular mass of this protein is within the expected range for a sugar transporter, it was considered to be a likely candidate for the glucose/xylose-H⁺ symporter. This hypothesis was supported further by the fact that binding of D-[¹⁴C]glucose to a protein with a similar molecular mass was observed upon incubation of gel slices with labelled sugar [22], performed immediately after the separation by SDS/PAGE (7.5% polyacrylamide gel, with 0.23% bisacrylamide) of membrane

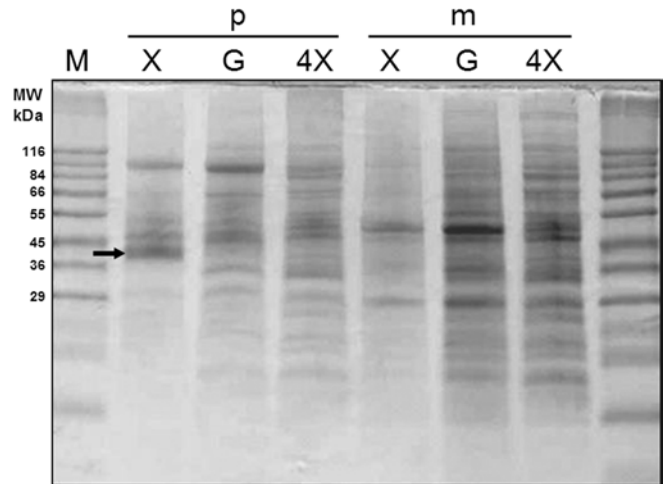


Figure 3 Tricine SDS/PAGE (10% polyacrylamide gel, with 0.3% bisacrylamide) of plasma- and mitochondrial-membrane proteins isolated from *C. intermedia* PYCC 4715 cells cultivated in Verduyn medium containing different concentrations of xylose or glucose as the sole carbon and energy source

The carbon sources were: 0.5% (w/v) xylose (X), 2% (w/v) glucose (G) or 4% (w/v) xylose (4X). The gel was stained with Coomassie Blue. M, molecular mass marker (Sigma-Aldrich, Wide Range); p, plasma membranes; m, mitochondrial membranes.

proteins from *C. intermedia* grown in low xylose concentrations (results not shown).

Cloning of the complete cDNA encoding the putative xylose-H⁺ symporter

The membrane protein identified in the SDS/polyacrylamide gel was subject to N-terminal sequencing, following preparative SDS/PAGE (7.5% polyacrylamide, with 0.23% bisacrylamide) and transfer to a PVDF membrane. This yielded a sequence of 15 amino acids (Figure 4). Two degenerate primers were designed based on this sequence and used for the amplification of the cDNA by RACE, using total RNA samples from cells cultivated in 0.5% (w/v) xylose. The 3'-RACE reaction was expected to yield nearly the complete cDNA, because the primer design was based on the N-terminal sequence of the protein. Indeed, an amplicon of 1.7 kb was obtained by 3' RACE and was subsequently cloned and partially sequenced. The encoded protein exhibited the features characteristic of a putative sugar transporter. Northern blot analysis subsequently showed that the respective gene was highly expressed in cells cultivated in 0.5% (w/v) xylose, whereas no mRNA could be detected in cells grown in 2% glucose (Figure 5). This is in line both with previous observations concerning the regulation of glucose- and xylose-H⁺-symport activity in *C. intermedia* PYCC 4715 and with the expression pattern obtained by SDS/PAGE (Figure 3). The 5' end of the cDNA was obtained by 5' RACE and found to encode one more amino acid at the N-terminus (the initiation methionine) and to include, in addition, a leader sequence of either 28 or 31 nucleotides. This indicates that two transcription start sites are



Figure 4 Amino acid sequence of the N-terminal region of Gxs1p and the degenerate primers used for the 3' RACE

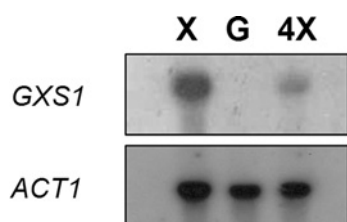


Figure 5 Northern blot analysis of *GXS1* expression in *C. intermedia* PYCC 4715 cells cultivated in different carbon sources

The carbon sources (w/v) were 0.5% xylose (X), 2% glucose (G) or 4% xylose (4X). Northern blots were probed with a *GXS1*-gene-specific probe and, subsequently, with an actin-gene-specific probe, as a loading control.

active. The novel gene, encoding a protein of 522 amino acids, was named *GXS1*.

Functional expression in *S. cerevisiae*

To confirm that the novel transporter protein encoded by the *GXS1* gene was involved in glucose and xylose transport, the complete cDNA was engineered for expression in *S. cerevisiae*, using *S. cerevisiae* promoters. A high-copy-number vector (pMA91) containing the *PGK1* promoter and terminator regions was used to clone the *GXS1* cDNA, whereas a different chimaeric *GXS1* gene driven by the *HXT7* constitutive truncated promoter was cloned both in the high- and low-copy-number vectors (YEplac 195 and YCplac 111 respectively).

The three plasmids were subsequently used to transform *S. cerevisiae* strain TMB 3201, generating strains MJY2–MJY5. Both high-copy-number plasmids (strains MJY2 and MJY3) were able to complement the growth defect of strain TMB 3201 on a solid medium containing glucose, but growth on xylose was hardly detectable (results not shown). Strain MJY4, carrying a low-copy-number plasmid expressing solely the *GXS1* gene driven by the *HXT7* promoter, grew very poorly on glucose and not at all on xylose solid media (results not shown), suggesting that complementation required stronger expression of the gene. Strain MJY2, carrying the *GXS1* gene driven by the *HXT7* promoter on a multicopy vector, was used to assay xylose- and glucose-proton-symport activity. The addition of D-glucose or D-xylose (6.7 mM) to an aqueous cell suspension of strain MJY2 elicited a pH increase in both cases, indicating the presence of a sugar- H^+ transport system (Figure 6). This shows that the *GXS1* gene encodes a sugar- H^+ symporter able to accept both glucose and xylose as substrates.

The *GXS1* gene supports growth of the *S. cerevisiae* *HXT*-null strain in glucose medium as well as the *GXF1* gene, when expressed from the same vector (Table 1, strains MJY1 and MJY2). However, when the levels of expression are higher, as is presumed to be the case for strain MJY3, the specific growth rate drops significantly. This could be due to the energetic burden imposed by the active transport system and/or to a deleterious effect of the heterologous expression of the Gxs1 protein itself, as is often the case for membrane proteins. To distinguish between these possibilities, we constructed a strain (MJY5) carrying both of the *C. intermedia* transporters. This strain could grow as well as strains MJY1 and MJY2, favouring the first hypothesis. However, very high expression of Gxs1 protein slightly affects the growth rate of strains MJY3 and MJY5 in maltose and that reduction cannot be ascribed to the energy spent with the symport mechanism.

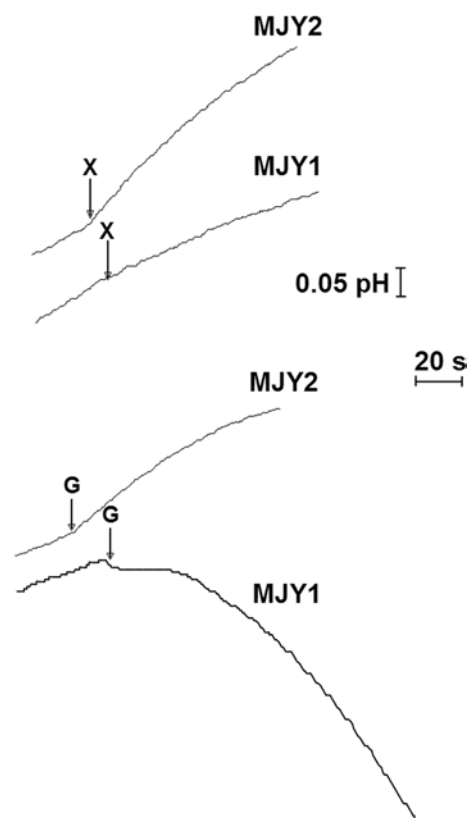


Figure 6 Increases in pH elicited by the addition of xylose (upper panel) or glucose (lower panel) to aqueous cell suspensions of *S. cerevisiae* strains MJY1 and MJY2 cultivated on glucose

Both sugars were added to a final concentration of 6.7 mM. The arrows indicate the times of sugar addition. X, xylose; G, glucose.

Table 1 Specific growth rates of *S. cerevisiae* strains carrying *C. intermedia* transporters

Strain	Specific growth rate (h^{-1})	
	Glucose (2%)	Maltose (2%)
TMB 3201	–	0.24
MJY1 (TMB 3201 + pGXF1)	0.15	0.23
MJY2 (TMB 3201 + pHXT7-GXS1)	0.16	0.24
MJY3 (TMB 3201 + pPGK-GXS1)	0.08	0.21
MJY4 (TMB 3201 + pHXT7-GXS1-s)	0.06	0.23
MJY5 (TMB 3201 + pGXF1 + pPGK-GXS1)	0.16	0.21

Kinetics of sugar transport by Gxs1

Sugar-uptake experiments with D- $[^{14}C]$ xylose and D- $[^{14}C]$ glucose were performed using strain MJY2 expressing the *GXS1* gene alone (Figure 7). The V_{max} values for glucose and xylose transport in this strain [0.26 ± 0.02 and 0.39 ± 0.09 mmol $\cdot h^{-1} \cdot (g \text{ of dry mass})^{-1}$ respectively] were one order of magnitude lower when compared with the V_{max} determined in strain MJY1 expressing the glucose/xylose facilitator Gxf1. Nevertheless, the estimated K_m values for xylose transport were similar to those determined in *C. intermedia* cells (0.4 ± 0.1 mM).

It was also confirmed that the affinity for glucose was higher than for xylose. However, the weak activity prevented an accurate estimate of the K_m value for glucose transport. For strain MJY5, expressing both *C. intermedia* transporters, a K_m of 0.2 ± 0.1 mM

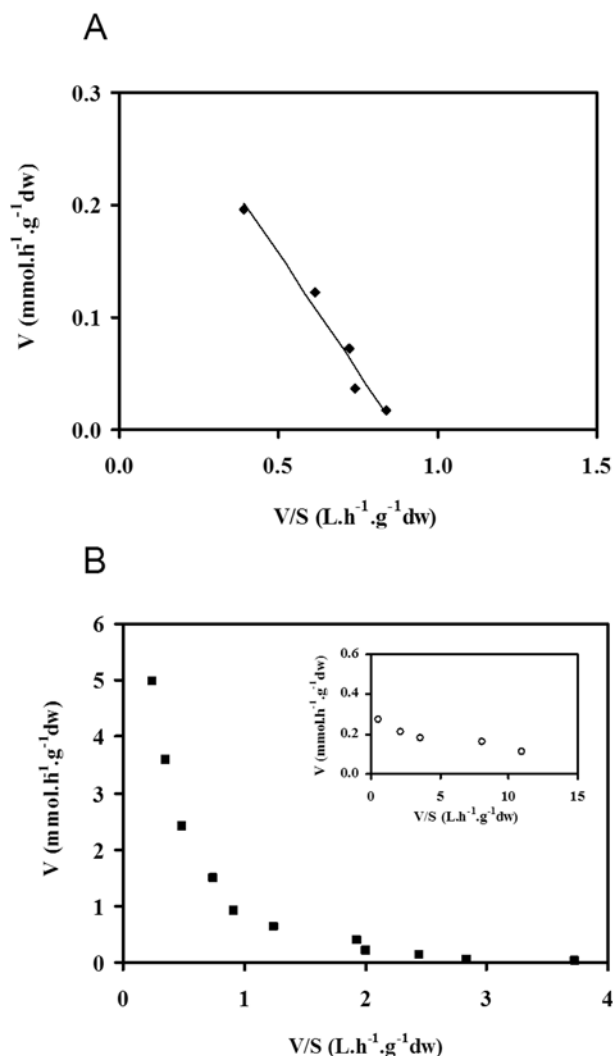


Figure 7 Glucose and xylose uptake mediated by Gxs1p and Gxf1p

Eadie-Hofstee plots of initial uptake rates of (A) D-[¹⁴C]xylose in strain MJY2, and (B) D-[¹⁴C]glucose in strains MJY5 and MJY2 (inset). dw, dry weight.

was obtained, a value that resembles that found in *C. intermedia* cells. However, when the kinetics of D-[¹⁴C]glucose transport were determined in strain MJY2, expressing only *GXS1*, a K_m of 0.012 ± 0.004 mM was estimated. It should be noted that MJY2 is devoid of the facilitated-diffusion system and that this may be reflected in estimates of the kinetic parameters. The very low substrate-concentration data points (Figure 7, MJY5 compared with MJY2), which are the least accurate, contribute more to the K_m determination.

Gxs1 belongs to a family of fungal monosaccharide-H⁺ symporters

The Gxs1 protein was predicted to have 12 transmembrane domains (TMHMM Server v. 2.0, <http://www.cbs.dtu.dk/services/TMHMM-2.0/>) and shows the highest similarity with uncharacterized putative sugar transporters from the yeasts *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Candida albicans* [NCBI (National Center for Biotechnology Information) protein accession numbers CAG86664, CAG81819 and EAL01541 respectively]. It is also quite similar to the MSTA protein of *Aspergillus niger*, which was recently shown to be a monosaccharide-H⁺

symporter with a broad substrate specificity [23]. This MSTA protein, in turn, bears high resemblance to the as-yet-uncharacterized transporters in the ascomycetous filamentous fungi *Neurospora crassa* and *Magnaporthe grisea*.

DISCUSSION

Plasma-membrane proteins can make up as much as a quarter of the total protein content of a yeast cell, playing a crucial role in the relationship between the cell and its environment. They can function as sensors of the external conditions, transducing important information to the cytoplasm and intracellular compartments and, more frequently, as transporters of solutes into the cell. In spite of their relative importance and abundance, functional characterization of integral membrane proteins in yeast and fungi clearly lags behind that of their soluble counterparts, probably because experimental handling of this type of protein poses additional difficulties. This also applies to our lack of knowledge, at the molecular level, of xylose transporters in yeast. Whereas XDH and XR from the xylose fermenter *P. stipitis* have long been identified, and were successfully expressed in *S. cerevisiae*, all attempts to isolate the cognate high-affinity xylose transport system(s) were unsuccessful. This stands in the way of a proper assessment of the impact of improved xylose transport on the fermentative capacity of currently available recombinant xylose-utilizing *S. cerevisiae* strains.

C. intermedia PYCC 4715 transports xylose with high efficiency [8], which prompted us to isolate the encoding genes for heterologous expression in *S. cerevisiae*. The first approach used was based on the possibility that the *C. intermedia* genes would complement the growth defect on xylose of an *S. cerevisiae* strain that was devoid of glucose and xylose transporters, using a *C. intermedia* genomic library. This approach led to the isolation of the gene encoding the *C. intermedia* glucose/xylose facilitator. The novel transporter protein (Gxf1) exhibited considerable sequence identity with the Sut1–Sut3 transporters found in *P. stipitis* and had an affinity for xylose of the same order of magnitude (approx. 50 mM compared with 50–150 mM; the present study and [7]).

Isolation of the glucose/xylose-proton symporter required a different strategy, based on differential expression of the protein in cells grown on different carbon sources. Starting with plasma-membrane preparations, it was possible to identify, by SDS/PAGE, the first high-affinity xylose/glucose symporter from yeast (Gxs1) and to clone its cDNA. The transporter properties of the protein, first suggested by the pattern of transcriptional regulation of the respective gene by the carbon source, were confirmed by functional expression of the complete cDNA in *S. cerevisiae* TMB 3201. The transformants expressing Gxs1 cDNA were able to grow on solid YNB medium containing glucose as the sole carbon and energy source. However, growth required a strong promoter and a high-copy-number vector, indicating that strong expression of the gene is necessary to allow growth. This may explain why it was not possible to isolate the gene by functional complementation, because its expression in the genomic library is driven by its own *C. intermedia* promoter, which is not expected to work as efficiently in *S. cerevisiae*.

None of the *C. intermedia* transporters was able to support vigorous growth of the recipient *S. cerevisiae* strain on xylose. It is noteworthy that the same recipient strain expressing only Hxt7, the best *S. cerevisiae* xylose transporter [9], also grows poorly on xylose. Our results warrant further investigations for the development of efficient bioethanol production processes from lignocellulosic materials.

There was evidence indicating that expression of the heterologous protein Gxs1 caused mild inhibition of growth of the *S. cerevisiae* strain used in this study. This negative impact can be inferred from the decrease in specific growth rate on maltose observed for some of the strains expressing the *C. intermedia* Gxs1 protein, when compared with the parental wild-type strain. In contrast, expression of the facilitator protein Gxf1 did not alter the specific growth rate of the parental strain on maltose. Moreover, we determined a very low V_{\max} value for Gxs1-mediated xylose uptake, which may also reflect cellular problems concerning the heterologous expression of the protein.

Characterization of Gxs1 and comparison of its amino acid sequence with those available in public databases led to the identification of closely related, uncharacterized until the present study, putative sugar transporters from three other yeast species (*D. hansenii*, *C. albicans* and *Y. lipolytica*). *D. hansenii* and *C. albicans* are known to produce xylose-H⁺ symporters, whereas *Y. lipolytica* is able to grow on xylose but the biochemical characteristics of sugar transport had not previously been investigated. In addition, Gxs1 also shares a high degree of identity with several putative sugar transporters from ascomycetous filamentous fungi, such as MSTA, a recently characterized broad-specificity sugar-H⁺ symporter from *A. niger* [23]. Hence a novel subgroup within the fungal sugar transporter family, which includes monosaccharide-H⁺ symporters with overlapping substrate specificity, seems to be emerging. This will give a new impetus to the identification of protein regions that determine the mechanism of transport (H⁺ symport versus facilitated diffusion) as well as substrate specificity. Further studies, involving additional members of this subfamily of high-affinity xylose transporters, are expected to yield an effective molecular tool to improve xylose transport in *S. cerevisiae*.

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